Detection of Viral DNA Within Skin of Healed Recurrent Herpes Simplex Infection and Erythema Multiforme Lesions

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The polymerase chain reaction (PCR) was used to detect HSV DNA in genomic DNA extracted from skin biopsies obtained from healed skin of five patients with hyperpigmented macules following recurrent cutaneous HSV infections and from eight patients with HSV-associated erythema multiforme (EM). A 92-bp HSV-1 DNA fragment was found in all the skin biopsies from the site of recurrent HSV infection and in five of eight (62%) biopsies from the EM patients. Virus DNA was not found in tissues distant from the site of HSV recurrence or from a patient without a history of HSV infection. These findings confirm the presence of HSV in healed skin from the site of recurrent HSV disease and are consistent with the concept that HSV is involved in EM pathogenesis. *J Invest Dermatol 98:68–72, 1992*

Herpes simplex is a common disease afflicting primarily the skin and mucous membranes. Following primary infection the virus enters an extended intracellular dormant phase during which the patient is without overt clinical disease and the virus is inaccessible to antiviral therapy. During this phase, designated latency, the virus is known to persist within neurons. The molecular basis of latency, and the genome functions involved in establishing and maintaining latency, have not yet been clarified. However, it is well known that virus is intermittently reactivated to an infectious state causing recurrent lesions at peripheral sites such as the skin or eye. It is generally believed that virus reactivation occurs in the ganglia and the newly generated infectious virus migrates down the axon to the skin where it replicates to produce a visible lesion. Extraneuronal maintenance of HSV has been postulated. However, in general it has been difficult to demonstrate infectious virus in intact skin during latency [1].

Erythema multiforme (EM) is a polymorphous eruption composed of macules, papules, bullae, and targetoid lesions that are symmetrically distributed but have a propensity for the distant extremities and oral mucosae. It can follow a HSV infection and, like HSV, is a self-limited, often recurrent disorder. It is estimated that 15–63% of EM cases are secondary to HSV [2,3] and that most cases of idiopathic EM are related to subclinical HSV infection [4,6]. However, viral cultures of EM lesions are usually negative [7] and the exact role of HSV in EM pathogenesis is still unclear.

A recently developed technique, the polymerase chain reaction (PCR) [8] allows for selective amplification of a given sequence of exogenous or endogenous DNA. The ability to amplify DNA and the sensitivity of the PCR procedure (PCR can detect a single copy of a gene in 10^5 cells) facilitate the detection of rare pathogenic DNA sequences in the presence of vast excesses of host nucleic acids. The studies described in this report used PCR amplification and Southern blot hybridization to determine whether intact skin from patients with a history of recurrent HSV infections or EM eruptions harbors HSV DNA sequences.

MATERIALS AND METHODS

Experimental Cases Punch skin biopsies were obtained from five patients with hyperpigmented macules following recurrent cutaneous HSV infections (Table I) and eight cases of HSV-associated EM (Table II). In three of the five HSV cases, oral acyclovir had been administered (200 mg, t.i.d.) before the biopsy was performed. All specimens were obtained 7–72 d after clinical resolution of the HSV lesions. As a positive control, biopsies were obtained from two cases of disseminated primary cutaneous HSV infections. As negative control, biopsies were obtained from a normal individual without a history of HSV infection and from two facial nevi from one of the HSV patients whose HSV lesions are located at a site distant from that of the nevi (Table I, case 3).

Virus Isolation All specimens that were assayed for infectious virus were inoculated on duplicate MRC-5 cultures. Virus typing was by immunofluorescent staining with specific monoclonal antibody (Syva, Microtrak) [9].

Treatment of Specimens and DNA Extraction The specimens to be tested by PCR were either formalin-fixed paraffin-embedded blocks or unfixed frozen tissue. One to five 5–6 μM thick section(s) were used for DNA extraction from the paraffin embedded tissues following the method of Sharma et al [10]. Briefly, the tissue sections were deparaffinized, vortexed, and pelleted by centrifugation. Specimens were washed twice with ethanol and resuspended in digestion buffer (50 mM Tris-HCl, pH 8.0; 1 mM EDTA, pH 7.5; 0.45% Tween 20; 0.45% NP40 and 200 μg/ml

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Abbreviations:
CMI: cell-mediated immunity
EM: erythema multiforme
G + C: guanine + cytosine
HSV: herpes simplex virus
LAT(s): latency associate transcript(s)
PCR: polymerase chain reaction

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Table I. Case Summaries of Patients with Scar or Pigmented Lesion After Cutaneous HSV Infection

Case Number	Age/Sex	Site	Acyclovir, 200 mg t.i.d. (days) Prior to Biopsy	Days Post Vesicular	Herpes Simplex Polymerase Chain Reaction
1	39/F	Finger	–	17	+
2	49M	Penis	16	30	–
3	42/M	Buttock	180	7	+
		Facial nevus 1		NA	–
		Facial nevus 2		NA	–
4	40/F	Thigh	90	72	+
5	57/M	Buttock	0	22	+
6	Primary HSV				+
7	Primary HSV				+
8	Normal control				–

proteinase K). Frozen tissues were minced in 0.5 ml of digestion buffer. Tissues were digested at 37°C for 12–24 h and DNA was extracted with phenol and chloroform, precipitated in absolute ethanol, and washed according to standard techniques. For rapid DNA purification, the samples were heated to 100°C in a water bath for 7 min and centrifuged and the supernatants were used. Total HSV genomic DNA extracted in parallel from HSV-2 (G strain) infected HEp-2 cells (24 h post-infection) was used as a positive control. Preliminary experiments showed no difference in positive yields between frozen and paraffin-embedded specimens.

PCR Procedure To amplify DNA we used oligonucleotide primers 5′CATCACCGACCCGGAGAGGGAC and 5′GGGCCAGGCGCTTGTTGGTGTA that bracket a 92 base pair (bp) segment of the HSV DNA polymerase gene that is common to both HSV-1 and HSV-2 with no cross-reaction with other herpes viruses [11]. Considerations in the selection of the appropriate primers included primer length, guanine plus cytosine (G + C) base content, and intrastrand primer complementarity. These particular primers were previously used in PCR amplification of DNA from EM tissues [6,11] and from skin tissues obtained from patients with HSV infection [11].

To verify the quality of tissue DNA extraction, oligonucleotide primers 5′-GAAGAGCCAAGGACAGGTA and 5′-CAACTTCATCCACGTTCACC corresponding to the human β-globin gene were assayed in parallel as described [12]. These primers amplify a 110-bp DNA sequence [12]. One hundred microliters of reaction mixture used in the PCR analysis contained 10 μg of sample DNA, 5 μl of 20X reaction buffer (New England Biolabs, Boston, Mass), 0.5 mM of dNTP (Perkin-Elmer Cetus Instruments, Norwalk, Conn), 20 pmole of each oligonucleotide primer, and 2.5 U of replinase (New England Nuclear). Equivalent amount of salmon testes DNA (Sigma, St. Louis, Mo) was used as a negative control. For each tissue specimen the PCR assay was performed using half of the extracted DNA with the HSV primers and the other half with the β-globin primers. The PCR reaction was performed in a DNA thermocycler (Perkin-Elmer Cetus) with 40 cycles of denaturation at 94°C for 2 min, followed by annealing at 52°C for 2 min, then elongation at 72°C for 3 min.

Confirmation of PCR Product Identity by Southern Blotting Following PCR amplification, the test mixture was electrophoresed on 8% acrylamide gel before staining with ethidium bromide for visualization of DNA. ϕX174 DNA (Bethesda Research Laboratories, Bethesda, Md) cut with HaeII was used as a size marker in all gels. The DNA was electrophoretically (85 V, 2 h) transferred from the gel to a Genescreen filter (New England Nuclear) and fixed by baking at 80°C for 4 h. It was prehybridized at 60°C overnight in a prehybridization mixture (0.1% polyvinylpyrroedine, 0.01% BSA, 0.1% Ficoll) with 150 μg/ml of denatured salmon testes DNA. Hybridization at 60°C overnight was performed with [γ^{32}P]-end labeled oligonucleotide probe 5′-TTTGTCCTCACCGCCGAACT (1×10^8 cpm/μg). This probe corresponds to the internal region of the expected DNA polymerase amplified gene. The filters were washed in succession with 2 × SSC twice at room temperature for 15 min each, 2 × SSC with 1% sodium dodecyl sulfate twice at 60°C for 30 min each, and then 0.5 × SSC twice for 30 min each. Autoradiography with an intensifying screen was done at 25°C with Kodak X-AR film [13].

RESULTS

The skin from three patients with a history of HSV recurrent disease was obtained from the site of the recurrent lesions identified by a minimal discoloration. In the other positive case the site was identified by the patient. The skin surface was completely healed in all these instances and the biopsy was obtained at 7–72 d post-healing. Two of the four positive patients had received acyclovir for 90–180 d prior to the biopsies. In one patient (Table I, case 3), two normal facial nevi were also studied, as was skin from a patient without a history of HSV infection. In one of the patients (Table I, case 5) 6 mg prednisolone had been administered intravenously for 3 d prior to biopsy. This patient, whose lesion healed 15 d earlier, had cancer

Table II. Summary of Data on Erythema Multiforme Patients Whose Cutaneous Lesions Were Studied by PCR for HSV DNA

Patient	Age/Sex	Biopsy Site	Duration of of Recurrent HSV Infection	Duration of Acyclovir Prophylaxis Prior to Biopsy	Days Post EM Outbreak at Biopsy Site	Herpes PCR
1	29/F	Elbow	6 years	10 months	14[b]	+
2	31/F	Arm[a]	1 year	0	5	–
3	16/M	Hand	21 days	0	7	+
4	33/M	Arm	21 days	0	3	+
5	47/M	Arm	16 years	0	16	+
6	47/F	Arm[a]	9 months	14 days	10	–
7	72/F	Finger	6 years	3 years	3	–
8	36/M	Thorax	22 days	0	22[b]	+

[a] Portion of biopsy found to be histologically compatible with EM.
[b] Cutaneous lesion clinically healed.

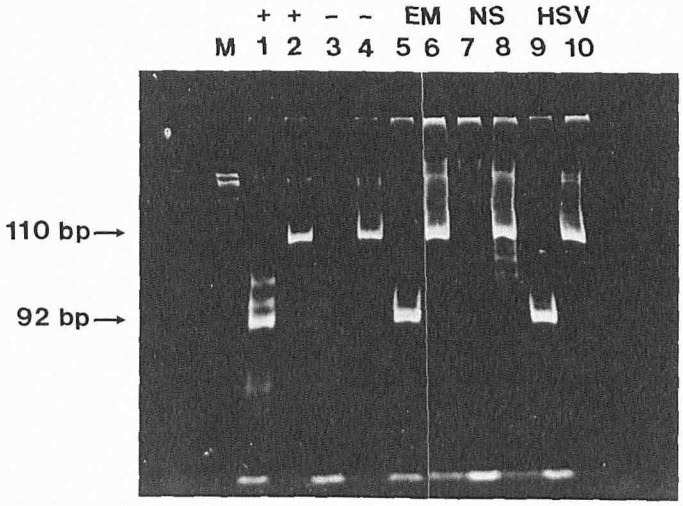

Figure 1. A representive gel of PCR data obtained in these experiments. *Arrows* in the left margin indicate HSV DNA (92-bp fragment) and β-globin DNA (110-bp). The lanes are labeled as follows: *M,* ϕX174 molecular size markers; *lanes 1 and 2,* patient 6 (Table I) with active HSV lesions; *lanes 3 and 4,* facial nevus DNA from patient 3 (Table I); *lanes 5 and 6,* a representive case of erythema multiforme (EM); *lanes 7 and 8,* normal skin (NS); *lanes 9 and 10,* HSV-2 infected HEp-2 cells. DNA samples were divided equally and specimens in the *odd-numbered lanes* were assayed with HSV primers, whereas those in the *even-numbered lanes* were assayed with the β-globin primers.

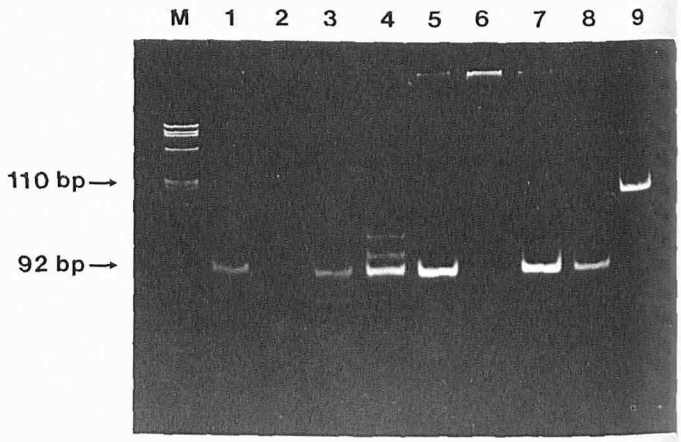

Figure 2. Results of PCR amplification of the 92-bp fragment of the HSV DNA polymerase gene *(lanes 1–8)* and the 110-bp fragment of the human β-globin gene *(lane 9).*

of the lung, metastatic to the vertebral column with resultant cord compression. Culture-proved HSV lesions appeared in a dermatomal distribution corresponding to that of the cord compression. Although the biopsied dermatome had been free of lesions for 15 d, cutaneous 3-d old lesions were visible nearby. In none of the five patients studied were lesions apparent near the surgical site within 7–20 d after the biopsy procedure. HSV isolations were obtained from the two positive control patients with active HSV lesions (Table I) (cases 6 and 7). In both cases the isolates were typed as HSV-1.

On visualizing the DNA on gels with ethidium bromide, a band of the anticipated size for the amplified HSV DNA fragment (92 bp) was seen for patient 6 (Table I) with active HSV lesions (Fig 1, *lane 1*) but not for the normal control patient (Fig 1, *lane* 7). Bands of the anticipated size for the amplified β-globin DNA fragment (110 bp) were seen for both patients (Fig 1, *lanes 2 and 8*). These same bands were also seen for DNA extracted from HSV-2–infected HEp-2 cells (*lanes 9 and 10*). The HSV DNA band was also seen in an EM case that was also positive for β-globin (Fig 1, *lanes 5 and 6*) but it was not seen in the DNA from one of the facial nevi of the HSV positive patient 3 (Fig 1, *lanes 3 and 4*).

The same HSV DNA band was also seen in biopsies of hyperpigmented skin (at sites of recurrent HSV) from three of four patients with a history of recurrent HSV (Fig 2, *lanes 1, 3, and 4*). The fourth patient was negative for HSV (Fig 2, *lane 2*) but was positive for β-globin (Fig 2, *lane 9*). Two cases of EM (Fig 2, *lanes 7 and 8*) were both HSV positive. Figure 3, *lanes 1 and 2 respectively represent* the amplification of the HSV and β-globin sequences for the post-HSV skin of patient 5 (Table I). Another EM-positive case (8) is shown in Fig 3, *lanes 3 and 4* (HSV and β-globin primers, respectively). Southern blot hybridization with the probe covering the internal region of the amplified HSV sequence confirmed the identity of the 92-bp band as HSV specific (Fig 3*B*). All the results are summarized in Tables I and II.

All eight patients with EM had a history of prior HSV infections. Five of eight (62%) patients had active or healed lesions positive for HSV DNA. One of these (Table II, case 1) had received acyclovir (200 mg t.i.d.) for 10 months prior to the biopsy. Herpes DNA material was demonstrated in this patient in a lesion that had been clinically healed for 4 d. Another patient (Table II, case 8), whose lesion had been healed for 6 d, was also positive for HSV DNA.

DISCUSSION

The possibility that the HSV genome (or fragments thereof) is retained in extraneuronal tissue in the interim between recurrent disease has attracted much attention over the years. Maximal virus

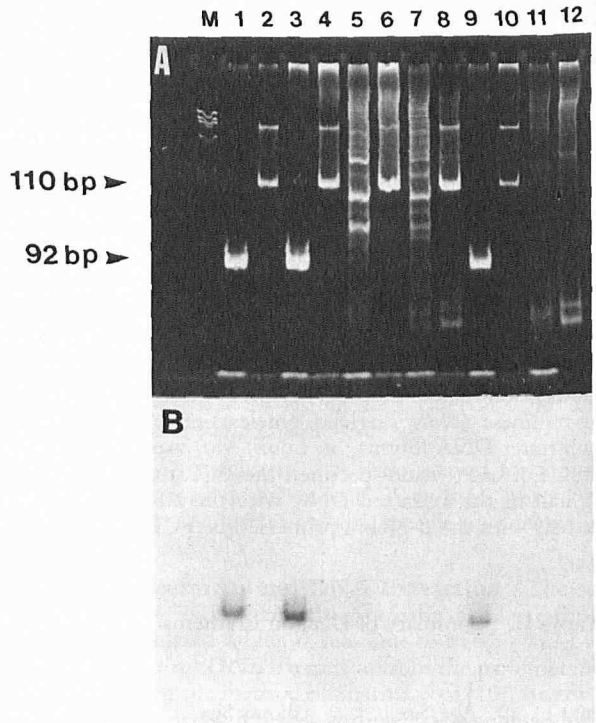

Figure 3. *A,* representative gel electrophoresis of PCR results. *Lane M,* ϕX174 molecular size markers. The *odd-numbered lanes* were amplified with HSV primers; the *even-numbered lanes* were amplified with β-globin primers. *lanes 1 and 2,* DNA from patient 5 (Table I); *lanes 3 and 4,* DNA from EM patient 8 (Table II); *lanes 5–8,* DNA from two normal nevi from case 3 (Table I); *lanes 9 and 10,* DNA from HSV-infected HEp-2 cells as positive control; *lanes 11 and 12,* salmon testes DNA as negative control. *B,* Southern blotting of the specimens in *A* using a probe covering the internal region of the expected HSV polymerase amplified gene. The lane designation is the same as in *A.*

titers, achieved prior to and at the time of the appearance of clinical recurrent symptoms, diminish as the lesion evolves and infectious virus has been difficult to isolate from clinically normal postlesional skin [1,14]. Dermal and corneal latency has been suggested based on i) virus recovery through co-culture; ii) detection of viral antigen by immunofluorescent staining or ELISA techniques [14–18], and iii) direct detection of HSV nucleotide sequences in latently infected animal and human herpetic corneas [15] by molecular hybridization assays. Furthermore, Hoyt and Billson [19] reported recurrent HSV in patients 7–10 d after blowout fractures had severed the nerve supply to the area where the lesions were prone to develop, suggesting that they arose from virus present in the skin at the time of injury. However, the difficulty encountered in demonstrating the presence of infectious virus in the human skin by conventional culture suggested that, if it is present, it must be at very low levels.

PCR offers a rapid, simple, sensitive, and specific test for HSV detection in skin and mucosa in the absence of overt lesions. It was successfully used to detect viral DNA in small punch skin biopsies from paraffin-embedded blocks of four patients with clinical signs and symptoms consistent with HSV [11] and in other patients with unusual manifestations of cutaneous herpes simplex [20,21]. However, these results were not directly correlated with clinical status and virus isolation.

PCR was also recently used by Brice et al [6] who reported the presence of HSV DNA in nine of 13 HSV-associated EM cases and in six of nine cases of idiopathic EM. Indeed, since the first report associating EM with HSV, support has accumulated for this association, based on clinical grounds. Viral cultures have been almost uniformly negative. However, virus particles were reported in one case using electron microscopy [7] and HSV-specific antigens were described in EM lesions using indirect immunofluorescent staining [22]. The histopathology of EM lesions supports the interpretation that cell-mediated immune (CMI) processes, possibly directed against HSV antigens, are present locally in the skin from EM lesions [23].

In this paper we present evidence indicating the presence of HSV DNA sequences in epithelial tissues at the site of pre-existing HSV lesions from clinically characterized patients and in epithelial tissues from HSV-associated EM lesions. The following variables were optimized in the PCR assay. With regard to primer design, 50% G + C content is required, and the number of complementary bases between the two primers needs to be minimized. In this way, "primer-dimer" formation is reduced, which may affect overall PCR efficiency [8]. Although the primers that we selected were used previously [11], we chose them because they match these requirements. The concentration of the primers, magnesium, Taq polymerase, and dNTP is also important. Also the annealing temperature was increased to 52°C. This is an optimal annealing temperature for this primer set under the specific experimental conditions used in these studies (data not shown), thereby resulting in a significant increase in PCR amplification efficiency and specificity [8,10].

After optimizing these parameters, we obtained a high specificity of the HSV primers based on the following criteria: i) a single amplification band in a size (92 bp) that was expected for these primers; ii) lack of an amplification signal in salmon sperm DNA and negative-control skin; iii) lack of an amplification signal in normal skin from a HSV patient but at a site distant from that of the HSV lesion; and iv) failure of our primers to elicit positive signals when tested against purified vaccinia virus DNA (data not shown). Unfortunately, we did not have skin samples from other viral infections for analysis in our PCR assay. Furthermore, we did not as yet test primers that are specific for other HSV DNA sequences. Most significantly, in this context are primers for the latency-associated transcripts (LAT) that are abundant in the latently infected human [24,25], rabbit [26], and murine [27] trigeminal ganglia and may be important for initiating and maintaining viral latency [28] or in HSV reactivation [29,30]. Such primers are presently under investigation in our laboratory in a prospective study of patients following primary HSV infection. In situ hybridization studies with HSV-specific oligonucleotide probes are also ongoing. They are designed to identify the cell type that harbors the HSV DNA sequences.

A crucial question is the association of the demonstrated PCR-amplified gene sequences with HSV extraneuronal latency and the etiology of EM. Marker rescue studies [31], in which human ganglia were infected with HSV mutants carrying specific markers, and studies investigating cloning of HSV-1 terminal fragments from DNA extracts suggested that defective HSV genomes may reside in ganglionic cells and most parts of the latent genome undergo rearrangement [32]. However, these reports focused on the trigeminal ganglia and did not address the question of a defective or rearranged latent gene in the skin. At least four possibilities can explain detection of HSV sequences in skin samples after resolution of active HSV disease: i) the DNA sequences may result from virus reactivation in the ganglia due to preoperative anxiety or stress from the local surgical procedure; ii) the DNA sequences could represent defective (non-functional) virus DNA sequences retained in the skin after acute or recurrent disease; iii) the sequences could represent a latent HSV gene; or iv) normal skin could possess a sequence similar to the probe used in these studies.

The negative results obtained in some of the patients, particularly in the facial nevi from the patient with HSV lesions on the buttock, argue against the last interpretation. Although we cannot fully exclude the possibility that stress or trauma may have reactivated the virus, this is not supported by the negative specimens obtained from some of our patients known to have had herpes, and the short time period required to obtain the surgical biopsy. Indeed, HSV is thought to spread along peripheral nerves at a speed of 2–10 mm/h [14]. Future compilation of additional data, using other primers such as LAT and HSV-RNA PCR amplification analysis after reverse transcriptase template formation [33], should provide more information about gene expression and function during latency. In this context it is of interest that skin obtained either from the scar or a post-infection granulomatous lesion within the dermatomal distribution of recent zoster infections did not contain viral DNA [34]. Thus, herpes zoster virus, in apparent contrast to HSV, does not seem to reside in cutaneous tissue during latency.

Our data confirm the findings of Brice et al [6] indicating that HSV DNA sequences persist in the skin and are present at the site of EM lesions. However, we have no data on the presence of viral antigens in the skin nor is it clear whether the HSV DNA persists between episodes of EM. It is also not known whether non-lesional skin in patients with HSV-associated EM has detectable HSV DNA. Prospective studies of biopsy specimens taken from lesional and non-lesional skin at different stages in the evolution of EM (and between recurrent episodes) using primers that represent various HSV genes should provide significant information on the relationship between HSV infection and EM development. The ability to demonstrate which cases of EM are associated with HSV should also help in patient management, particularly as regards the use of prophylactic acyclovir.

In this context it may be significant that we find HSV DNA persisting in the skin despite the continuous prophylactic administration of acyclovir. This finding is compatible with the clinical observation of disease recurrence following discontinuation of acyclovir therapy.

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